MECHANISM OF ETHANOL-INDUCED CHANGES IN LIPID COMPOSITION OF *ESCHERICHIA COLI*: PREFERENTIAL INHIBITION OF SATURATED FATTY ACID SYNTHESIS

By

THOMAS MARTIN BUTTKE

A DISSERTATION PRESENTED TO THE GRADUATE COUNCIL OF
THE UNIVERSITY OF FLORIDA
IN PARTIAL FULFILLMENT OF THE REQUIREMENTS FOR THE
DEGREE OF DOCTOR OF PHILOSOPHY

UNIVERSITY OF FLORIDA

1978

ACKNOWLEDGEMENTS

Le chemin est long du projet à la close.
Molière

The author would like to thank Dr. L.O. Ingram for his help and encouragement during the author's graduate career. Dr. Ingram's knowledge as well as his friendship has been invaluable and his willingness to share both is greatly appreciated.

Thanks are also due to Drs. J.F. Preston and C.M. Allen for donating their time and suggestions during the course of this research.

The author is especially grateful to his wife, Mary, for providing companionship during late night experiments, and for typing this dissertation. Her encouragement and good humor have contributed immeasurably towards the completion of this research.

Part of this work was performed while the author was the recipient of a fellowship from the Center for Gerontological Studies, and this support is gratefully acknowledged.

TABLE OF CONTENTS

	Page
ACKNOWLEDGMENTS	ii
LIST OF TABLES	iv
LIST OF FIGURES	v
ABBREVIATIONS USED	vi
ABSTRACT	vii
INTRODUCTION	1
EXPERIMENTAL PROCEDURE	4
RESULTS	13
DISCUSSION	47
REFERENCES	54
BIOGRAPHICAL SKETCH	57

LIST OF TABLES

Table		Page
1	Bacterial strains.....	5
2	Effect of ethanol on the fatty acid composition of newly synthesized phospholipids in strain TB4.....	14
3	Effect of incubation temperature on ethanol-induced changes in the fatty acid composition of newly synthesized phospholipids.....	17
4	Effect of fatty acid degradation on ethanol-induced changes in fatty acid composition.....	20
5	Effect of ethanol on the fatty acid composition of an unsaturated fatty acid auxotroph, strain K1060.....	25
6	Effect of ethanol on the incorporation of fatty acids into phospholipids.....	28
7	Effect of exogenous 16:0 on ethanol-induced changes in fatty acid composition in strain TB4.....	30
8	Effect of ethanol on the incorporation of exogenous 16:0 [^{3}H] and endogenous 16:0 [^{14}C] into the phospholipids of strain TB4.....	32
9	Effect of ethanol on the incorporation of 17:0 into the phospholipids of an unsaturated fatty acid auxotroph, strain K1060.....	33
10	Effect of ethanol on the fatty acids synthesized *in vitro* by a crude fatty acid synthetase.....	35
11	Effect of purification of fatty acid synthetase on ethanol-induced fatty acid changes *in vitro*.....	37
12	Effect of ethanol on the chain lengths of the fatty acid products synthesized *in vitro*.....	44
13	Effect of incubation temperature on the fatty acid products synthesized *in vitro*.....	46

LIST OF FIGURES

Figure		Page
1	Biosynthetic pathway for fatty acids in E. coli showing the location of the defects in fatty acid synthesis mutants.....	7
2	Effect of ethanol on free fatty acid synthesis and total lipid synthesis in strains LW1 and LW3.....	23
3	Effect of ethanol on the rates of synthesis of saturated and unsaturated fatty acids in vitro.....	40
4	Argentation chromatography of fatty acid products synthesized in vitro.....	42

ABBREVIATIONS USED

UFA	Unsaturated fatty acid
SFA	Saturated fatty acid
PFA	Polar fatty acid
ACP	Acyl carrier protein
16:0	Palmitic acid
16:1	Palmitoleic acid
17:0	Heptadecanoic acid
cis-Δ^{9}-18:1	Oleic acid
cis-Δ^{11}-18:1	cis-Vaccenic acid
Δ17	cis-9,10-Methylene hexadecanoic acid
20:2	Eicosadienoic acid
Δ19	cis-11,12 Methylene octadecanoic acid
MOPS	Morpholinopropane sulfonic acid

Abstract of Dissertation Presented to the Graduate Council of
the University of Florida in Partial Fulfillment of the Requirements
for the Degree of Doctor of Philosophy

MECHANISM OF ETHANOL-INDUCED CHANGES IN LIPID COMPOSITION OF ESCHERICHIA COLI: PREFERENTIAL INHIBITION OF SATURATED FATTY ACID SYNTHESIS

By

Thomas Martin Buttke

June 1978

Chairman: Lonnie O. Ingram

Major Department: Microbiology and Cell Science

Experiments were performed to determine the mechanisms by which ethanol induces fatty acid changes in Escherichia coli. Mutants deficient in various aspects of lipid metabolism were used to determine the mechanisms of ethanol interaction in vivo. Under conditions which uncoupled fatty acid synthesis from phospholipid synthesis, ethanol decreased the amount of saturated fatty acids synthesized, but had little effect on their incorporation into phospholipids. In the absence of fatty acid degradation and unsaturated fatty acid synthesis, E. coli still demonstrated ethanol-induced fatty acid changes, while the inhibition of total fatty acid synthesis eliminated this response. Experiments with the fatty acid synthetase enzymes in vitro supported the in vivo results. Fatty acid synthetase preparations from three levels of purification demonstrated a preferential inhibition of saturated fatty acid synthesis by ethanol, similar to that observed

in vivo. The fatty acid changes *in vitro* did not result from a total arrest of saturated fatty acid synthesis, nor were they due to a premature termination of saturated fatty acid elongation. These results indicate that ethanol-induced fatty acid changes in *E. coli* result from a preferential inhibition of the rate of saturated fatty acid synthesis.

INTRODUCTION

In a previous report Ingram demonstrated that Escherichia coli will alter its membrane fatty acid composition when grown in the presence of straight chain alcohols (Ingram, 1976). These alcohol-induced fatty acid changes are similar to those induced by changes in growth temperature (Marr and Ingraham, 1962). Short chain alcohols such as ethanol cause a decrease in the amounts of saturated fatty acids, similar to a shift down in growth temperature. Long chain alcohols such as hexanol induce changes analogous to a shift up in temperature, an increase in the proportion of saturated fatty acids (Ingram, 1976). Like temperature adaptation, these changes in fatty acid composition are independent of major changes in phospholipid composition (Ingram, 1977a). These results with alcohols have been extended to show that a wide variety of other lipophylic agents (organic solvents and food preservatives) also induce fatty acid changes in E. coli (Ingram, 1976). The diverse structures of these compounds as well as their lipophylic nature support the theory that these changes in fatty acid composition result from a general interaction with a hydrophobic site, rather than a result of specific catabolic processes (Ingram, 1977b). The intercalation of these agents into the membrane would be expected to alter membrane fluidity. The resulting changes in fatty acid composition suggest that the cell may be able to detect changes in membrane fluidity, per se, and compensate for the direct actions of drugs.

In view of the importance of ethanol, ethanol-tolerance and withdrawal syndrome in society, the ability of ethanol to induce dramatic changes in the fatty acid composition is of particular interest. A membrane whose lipid composition has been altered in response to ethanol could be considered as being adapted to the presence of the alcohol (tolerance), much as membrane lipid changes allow a membrane to adapt to growth temperature (Shaw and Ingraham, 1965). The removal of ethanol would cause additional changes in membrane organization which would be compensated for by a return to the original composition (withdrawal). Thus, an understanding of the mechanisms responsible for ethanol-induced changes in fatty acid composition in E. coli could provide a model for the development of experiments in mammalian systems.

The similarity of the changes in fatty acid composition induced by ethanol and temperature adaptation suggests that these processes may share a common mechanism. This regulation of fatty acid composition could be carried out at any of three levels: (1) fatty acid degradation; (2) fatty acid synthesis; and (3) phospholipid synthesis (Cronan and Vagelos, 1972). E. coli mutants blocked in one or more of these processes have proven useful in studying the control of thermal regulation of membrane lipid composition (Cronan and Gelmann, 1975). These same mutants were used in this study to elucidate the mechanisms by which ethanol induces membrane fatty acid changes in E. coli. The results of these experiments indicate that the ethanol-induced changes in fatty acid composition in vivo result primarily from the inhibition of saturated fatty acid synthesis.

Experiments were also conducted with the fatty acid synthesis enzymes *in vitro* to determine if the relative amounts of fatty acids synthesized *in vitro* were also affected by ethanol. In a fashion analogous to that observed *in vivo*, ethanol inhibited total fatty acid synthesis *in vitro* but with a preferentially greater inhibition of saturated fatty acid synthesis. Taken collectively, the results of both the *in vivo* as well as the *in vitro* experiments suggest that ethanol-induced fatty acid changes in *E. coli* result from a decreased availability of saturated fatty acids for phospholipid synthesis. Further, the decreased availability of saturated fatty acids reflects a preferential inhibition of saturated fatty acid synthesis by ethanol.

EXPERIMENTAL PROCEDURE

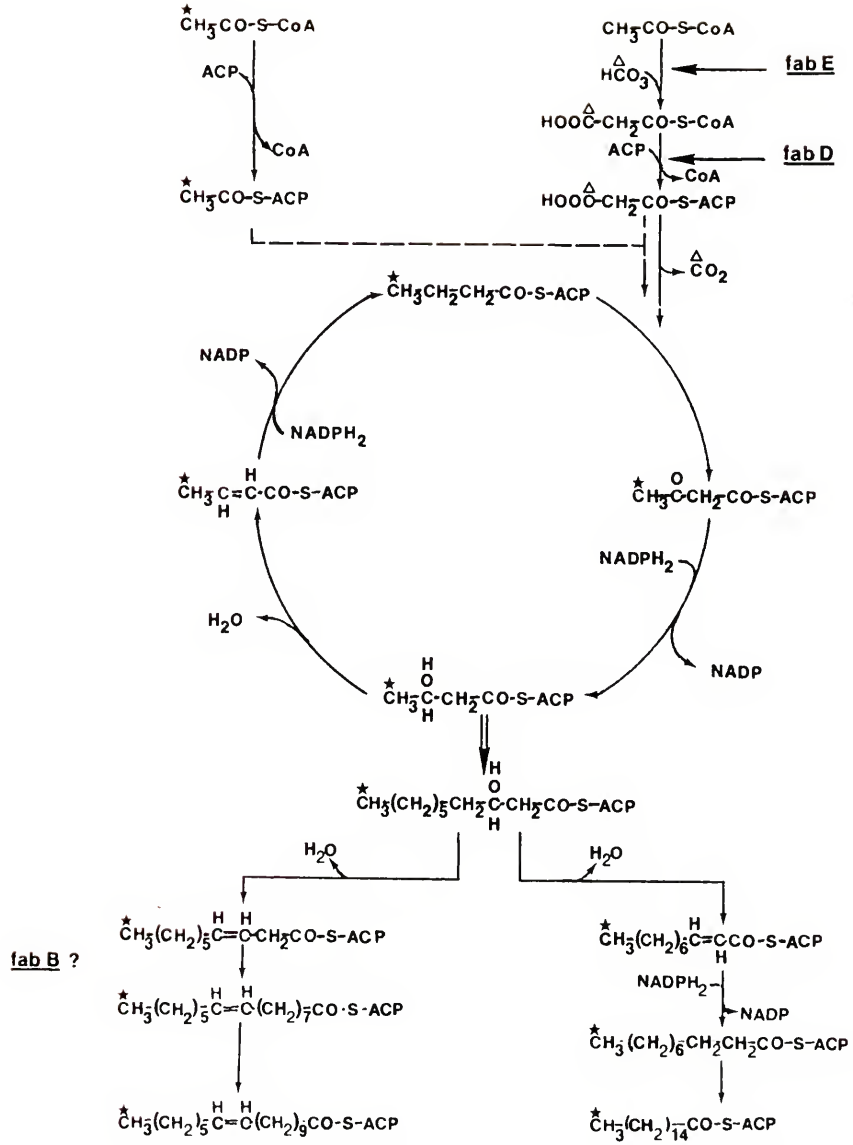
Bacterial Strains. The E. coli K12 derivatives used in this study are listed in Table 1. Figure 1 illustrates the biosynthetic pathway for fatty acids in E. coli, showing the location of the defects in fatty acid synthesis. Strain TB4 is derived from strain CSH2 and is defective in fatty acid degradation (fadE$^-$). Strain CSH2 was made proB$^-$ by mutagenesis with ultraviolet light followed by penicillin selection (Miller, 1972). A proB$^+$ fadE$^-$ lysate was made from strain L43 by the method of Marsh and Duggan (1972) using the Plvir (Cold Spring Harbor Laboratory), and this was used to introduce the fadE$^-$ gene into strain CSH2. Potential fadE$^-$ transductants were selected by their ability to grow without proline supplementation.

Growth Conditions. Unless otherwise indicated, all bacterial strains were grown in a gyrotory shaker at 30^{o}C. The standard medium used was E medium (Vogel and Bonner, 1956). Supplements differed for each strain. Strains CSH2, TB4 and L43 were grown with sodium succinate (0.2%), thiamine (0.001%), tryptophan (0.004%), and yeast extract (0.0005%). Strains LW1 and LW3 were supplemented with sodium succinate (0.2%), thiamine (0.001%), casein hydrolysate (vitamin free) (0.05%), and sn-glycerol-3-phosphate (0.01%). Glycerol-3-phosphate deprivation was performed by filtration as described by Cronan et al. (1975). For strains L8 and L48, the media was supplemented with glycerol (1.0%), thiamine (0.001%), monosodium glutamate (0.1%),

Table 1. Bacterial strains

Strain	Relevant Genotype	Relevant Phenotype	Source
CSH2	Wild Type	Wild type for fatty acid synthesis and fatty acid degradation	Cold Spring Harbor Laboratory
TB4	*fadE*$^-$	Prototrophic for fatty acid synthesis; defective in fatty acid degradation	This study
K1060	*fabB*$^-$ *fadE*$^-$	Defective in unsaturated fatty acid synthesis; defective in fatty acid degradation (Overath *et al.*, 1970)	D.F. Silbert and B.J. Bachmann
L43	*fadE*$^-$	Prototrophic for fatty acid synthesis; defective in fatty acid degradation (Silbert *et al.*, 1973)	D.F. Silbert
LW1	*fadE*$^-$ *plsA*$^-$ *glpD*$^-$	Defective in sn-glycerol-3-phosphate utilization, defective in fatty acid degradation; defective in glycerol catabolism (Cronan *et al.*, 1975)	J.E. Cronan, Jr.
LW3	*fadE*$^-$ *gpsA*$^-$ *glpD*$^-$	Defective in sn-glycerol-3-phosphate synthesis; defective in fatty acid degradation; defective in glycerol catabolism (Cronan *et al.*, 1975)	J.E. Cronan, Jr.
L8	*fabE*$^-$	Temperature-sensitive for fatty acid synthesis (Semple and Silbert, 1975)	B.J. Bachmann
L48	*fabD*$^-$	Temperature-sensitive for fatty acid synthesis (Silbert *et al.*, 1976)	B.J. Bachmann

Figure 1. Biosynthetic pathway for fatty acids in *Escherichia coli* showing the location of the defects in the fatty acid synthesis mutants.


CH3CO-S-CoA
ACP
CoA
CH3CO-S-ACP
CH3CO-S-CoA
HCO3
fab E
HOOC-CH2CO-S-CoA
ACP
fab D
CoA
HOOC-CH2CO-S-ACP
CO2
CH3CH2CH2CO-S-ACP
NADP
NADPH2
CH3C=CHCO-S-ACP
CH3COCH2CO-S-ACP
NADPH2
NADP
H2O
CH3CHOCH2CO-S-ACP
CH3(CH2)5CH2COCH2CO-S-ACP
H2O
H2O
fab B ?
CH3(CH2)5C=CCH2CO-S-ACP
CH3(CH2)5C=C(CH2)7CO·S-ACP
CH3(CH2)5C=C(CH2)9CO-S-ACP
CH3(CH2)6C=CCO-S-ACP
NADPH2
NADP
CH3(CH2)6CH2CH2CO-S-ACP
CH3(CH2)14CO-S-ACP

yeast extract (0.0001%), Brij 58 (0.4%), potassium palmitate (0.001%) and potassium oleate (0.001%). Cell growth was monitored by measuring the absorbance at 550 nm. using a Beckman model 25 spectrophotometer. Cultures were routinely used in the experiments at a cell density of $2x10^8$ cells/ml (0.36 absorbance units at 550 nm.) to insure that cells were in log phase.

Cells grown for in vitro fatty acid synthesis studies were grown into late log phase (0.70 absorbance units at 550 nm.) in Medium 63 mineral salts (Miller, 1972) supplemented with glucose (0.2%), thiamine (0.001%), tryptophan (0.02%) and casamino acids (vitamin free; 0.1%). These cells were also grown in a gyrotory shaker, except the incubation temperature was 37^oC.

Preparation of Fatty Acid Synthetase Enzymes. Cells grown into late log phase were harvested by centrifugation and washed once with 10 mM phosphate buffer pH 7.0. The cells were disrupted by passage through a French pressure cell (12,500 psi) and centrifuged at 5,000 rpm for ten minutes to remove unbroken cells. The supernate contained the fatty acid synthesis enzymes (Lennarz et al., 1962) and is called the "crude" enzyme preparation. A membrane free enzyme preparation was obtained by centrifuging the "crude" enzyme preparation at 100,000 x g for two hours to pellet the membranous material. The supernate which contained the fatty acid synthesis enzymes after this step was referred to as the "100 Kg" preparation. This preparation was further purified by ammonium sulfate fractionation as described by Lennarz et al. (1962). Ammonium sulfate was added to the "100 Kg" preparation to 55% saturation and the precipitated protein was removed by centrifuging at 10,000 rpm for ten minutes. The

supernate was brought to 75% saturation with additional ammonium sulfate and the precipitated fatty acid synthetase enzymes were harvested by centrifugation. The resultant pellet was resuspended in 10 mM phosphate buffer, pH 7.0, containing 1 mM dithiothreitol, and frozen at -20°C in 0.2 ml aliquots.

In Vitro Fatty Acid Synthesis Assay. The conditions for assaying fatty acid synthesis in vitro were a modification of the procedure of Silbert (1976). Standard incubation mixtures contained MOPS buffer, pH 7.1 at 37°C (50 µmoles), dithiothreitol (1 µmole), $NADH_2$ (100 nmoles), $NADP^+$ (100 nmoles), glucose-6-phosphate (1 µmole), glucose-6-phosphate dehydrogenase (0.65 units), acetyl CoA (15 nmoles), acyl carrier protein (25 µg), and [2-^{14}C] malonyl CoA (30 nmoles; 3.8 µCi/µmole) in a final volume of 0.48 ml. The reaction was started by the addition of 100 µg of enzyme protein, and the reaction mixtures were incubated at 37°C in screw-cap tubes sealed with Teflon-lined caps. The reaction was quenched by the addition of 1 ml of 15% KOH in methanol, and the samples were heated for two hours at 70°C. The samples were acidified with 0.6 ml of 6N HCl, carrier fatty acids were added, and the mixture was extracted two times with pentane. Combined pentane extracts were washed twice with water, added to pre-weighed screw-cap tubes, and the pentane was evaporated under nitrogen at 45°C until one gram of pentane remained. Aliquots of 100 µl were removed for measuring total lipid synthesis, and the remainder was taken to dryness prior to transesterification. The labeled methyl esters were separated into saturated, unsaturated and polar fatty acids by argentation chromatography.

Analysis of Fatty Acids by Gas Chromatography. Cells were harvested by centrifugation, inactivated by the addition of 5% trichloroacetic acid, and extracted into chloroform-methanol as described by Kanfer and Kennedy (1973). For the separation of neutral lipids (includes free fatty acids) from phospholipids, washed lipid extracts were chromatographed on unactivated silica gel G impregnated glass-fiber paper (Gelmann ITLC-SG, Fisher Scientific Co., Pittsburg, Pa.) using solvent 1 as described by Freeman and West (1966). Phospholipids were eluted with chloroform-methanol (2:1), and transesterified using 2% H_2SO_4 in methanol, as described by Silbert et al. (1973a). The methyl esters were extracted into pentane and concentrated under N_2 prior to analysis. The conditions for gas chromatography have been described (Ingram, 1976).

Analysis of Radioactive Lipids. The incorporation of labeled acetate into fatty acids was assayed essentially by the procedure of Cronan and Wulff (1969). One milliliter samples of cells were added to tubes containing 10 μCi of [^{14}C] acetate (61 mCi/mmole) and incubated at 37°C for ten minutes. The reaction was stopped by the addition of chloroform-methanol (1:2) and extracted overnight. The resulting lipid extract was separated into two phases by the addition of chloroform and water; radioactive lipids were present in the lower (chloroform) phase. An aliquot (0.2 ml) was removed from the chloroform phase and counted to determine total lipid synthesis. The remaining radioactive lipids were transesterified as described above, and the radioactive methyl esters were analyzed by argentation chromatography. When the incorporation of [^{14}C] acetate into free (non-esterified) fatty

acids was measured, the lipid extract was separated into phospholipids and neutral lipids by thin layer chromatography using either the solvent system of Klein et al. (1971) or the two step procedure described by Cronan et al. (1975). Both the phospholipid and the neutral lipid fractions were eluted, transesterified and analyzed by argentation chromatography. Incorporation of exogenously supplied [^{14}C] or [^{3}H] palmitic acid and [^{3}H] oleic acids were performed in essentially the same fashion, except phospholipids were separated from neutral lipids by thin layer chromatography using the solvent diethyl ether-acetic acid (100:1). The phospholipid region was scraped into vials containing toluene scintillation solution (Omnifluor; New England Nuclear) and counted on a Beckman model 133 liquid scintillation counter. When the counter was adjusted for dual-label counting, the efficiency was 58.2% for [^{3}H] and 72.5% for [^{14}C].

Argentation Chromatography. Thin layer chromatography plates coated with silica gel G were impregnated with 10% aqueous silver nitrate and activated as described by Cubero and Mangold (1965). The labeled methyl esters were applied to the argentation plate in pentane and developed twice in toluene at -17^{o}C (Morris et al., 1967). Radioactive regions were scraped into vials and counted.

Reversed Phase Chromatography. The chain lengths of the saturated and unsaturated fatty acids synthesized in vitro were determined by reversed phase chromatography (Bergelson et al., 1964). Regions of argentation plates corresponding to saturated and unsaturated fatty acids were scraped and the methyl esters were eluted with pentane. The methyl esters were applied in pentane to silica gel G plates impregnated

with dodecane. Impregnation was achieved by ascending a 10% dodecane in light petroleum ether solution up a silica gel G plate and the petroleum ether evaporated. The solvent system used for development consisted of acetone-acetonitrile (1:1) saturated with dodecane (Bergelson et al., 1964). Labeled methyl esters were located by autoradiography using Kodak X-OMAT R film.

Chemicals. Heptadecanoic acid was purchased from Applied Science Laboratories Inc. (State College, Pa.). All other unlabeled fatty acids were obtained from the Sigma Chemical Co. (St. Louis, Mo.). [1-^{14}C] Acetate, [9,10(n)-^{3}H] oleic acid, and [1-^{14}C] palmitic acid are products of Amersham Corp. (Arlington Heights, Il.). [9,10(n)-^{3}H] Palmitic acid and [2-^{14}C] malonyl CoA were purchased from New England Nuclear (Boston, Ma.). Unlabeled malonyl CoA, acetyl CoA and dodecane are the products of Sigma Chemical Co. Acyl carrier protein (ACP) was purified by the procedure of Majerus et al. (1968) from one kilogram of E. coli K12 (Grain Processing Co., Muscatine, Iowa).

RESULTS

Effect of Ethanol on the Fatty Acid Composition of Newly Synthesized Phospholipids. Previous experiments have shown that growth in the presence of ethanol results in the synthesis of phospholipids rich in unsaturated fatty acids (Ingram, 1976). To determine if these changes represent a direct effect of ethanol on lipid synthesis (fatty acid synthesis and utilization) or a secondary effect mediated through such processes as enzyme induction or other alternatives in metabolism, we have examined the immediate effects of ethanol addition on newly synthesized lipids. Strain TB4 (*fadE*$^-$) was pulse-labeled with [^{14}C] acetate to measure the effects of various concentrations of ethanol on the incorporation of newly synthesized fatty acids into phospholipids.

The addition of ethanol caused an immediate increase in the abundance of newly synthesized unsaturated fatty acids which were incorporated into phospholipids (Table 2). This resulted in a dose-related increase in the UFA/SFA ratios, and is analogous to the changes previously observed in the bulk lipid composition (Ingram, 1976). High concentrations of ethanol (4% and 6%) strongly inhibited the synthesis and utilization of both unsaturated and saturated fatty acids, and this inhibition may be related to the "lipid poor" cells described earlier (Ingram, 1977). The ethanol-induced increase in the UFA/SFA ratio was immediately reversed by ethanol removal (washing),

Table 2. Effect of ethanol on the fatty acid composition of newly synthesized phospholipids in strain TB4

	[^{14}C] Acetate Incorporation (cpm)			
EtOH(%)	Total	Saturated	Unsaturated	UFA/SFA Ratio[c]
0[a]	14,400	4,100	10,300	2.5
1	8,800	1,700	7,200	4.2
2	9,300	1,400	7,900	5.6
3	8,600	1,100	7,500	6.8
4	2,600	360	2,300	6.4
6	1,600	350	1,300	3.7
3%, 16 hr[b]	4,800	900	3,900	4.3
No EtOH	10,800	2,800	8,000	2.9

[a]Cells were grown in the absence of ethanol. Samples were removed and added to tubes containing 10 μCi of [1-^{14}C] acetate (61 mCi/mmole) with various concentrations of ethanol. The samples were incubated for ten min. Fatty acids were separated using argentation chromatography.

[b]Cells were grown in the presence of 3% ethanol for 16 hr (with appropriate dilutions). The culture was split into two parts. One part was rapidly washed using membrane filtration and resuspended to its original volume. Samples were removed from both parts, pulsed with [^{14}C] acetate and analyzed by argentation chromatography.

[c]UFA, unsaturated fatty acid; SFA, saturated fatty acid

even after 16 hours of growth in the presence of 3% ethanol. The immediacy of action of ethanol on the UFA/SFA ratio of newly synthesized lipids coupled with its equally rapid reversibility provide evidence for the direct action of ethanol on lipid synthesis.

Pulse-labeling experiments typically yielded UFA/SFA ratios which were higher than those of bulk lipids. This probably resulted from the incorporation of newly synthesized fatty acids into phosphatidyl glycerol. In E. coli this phospholipid is enriched in unsaturated fatty acids and turns over rapidly during normal growth. Thus, the pulse-labeling studies may reflect both the de novo synthesis as well as the turnover of phosphatidyl glycerol which would give rise to the increased UFA/SFA ratios observed. However, the UFA/SFA ratios following the initial addition of ethanol (Table 2) were much higher than expected based upon determinations of bulk lipid composition (Ingram, 1976). During growth in the presence of ethanol, the ratios observed in log phase cells pulsed with [^{14}C] acetate approached that of bulk lipid, 7.1, 6.1, 5.8, and 4.4 after 0, 1, 2, and 16 hours, respectively. Thus the initial increase in the UFA/SFA ratio following the addition of ethanol represented an overcompensation in newly synthesized lipids which was subsequently decreased as the cells adjusted to the presence of ethanol.

Effect of Incubation Temperature on Ethanol-Induced Changes in the Fatty Acid Composition of Newly Synthesized Lipids. E. coli alters its membrane fatty acid composition in response to changes in growth temperature (Marr and Ingraham, 1962). A shift to a higher growth

temperature leads to an increase in the proportion of palmitic acid while a shift to a lower growth temperature leads to an increase in the proportion of unsaturated fatty acids. These changes in lipid composition are regulated both at the level of fatty acid synthesis (Cronan, 1975; Okuyama et al., 1977) and fatty acid utilization for phospholipid synthesis (Esfahani et al., 1969; Sinensky, 1971). We have shown that the addition of ethanol causes changes in fatty acid composition similar to a reduction in growth temperature. To further explore this relationship, we have investigated the simultaneous effect of ethanol addition and a shift in incubation temperature on the fatty acid composition of newly synthesized lipids. Samples from an exponential culture of strain TB4 grown at 30°C were incubated at 23°C, 30°C and 37°C, and pulse-labeled with [^{14}C] acetate in the presence and absence of 3% ethanol. Esterified fatty acids were separated by argentation chromatography, scraped and counted.

Strain TB4 altered its fatty acid composition (UFA/SFA ratio) in response to temperature (Table 3) as previously reported (Marr and Ingraham, 1962). This same trend was reflected among samples containing ethanol indicating that ethanol did not disrupt the normal thermoregulatory systems. The addition of ethanol resulted in an increase in the proportion of unsaturated fatty acids at all incubation temperatures. Thus the effects of ethanol and a shift down in temperature were roughly additive while the effects of ethanol and a shift up in temperature were opposing.

The increases in the proportion of unsaturated fatty acids in response to ethanol addition and a shift to lower temperature were

Table 3. Effect of incubation temperature on ethanol-induced changes in the fatty acid composition of newly synthesized phospholipids[a]

Incubation Temperature	Conditions	Fatty Acids (cpm)			UFA/SFA Ratio[b]
		Saturated	16:1	cis-Δ^{11}-18:1	
23°C	No EtOH	1,600	3,600	5,300	5.6
	3% EtOH	520	2,900	2,800	11.0
30°C	No EtOH	2,500	2,700	3,800	2.6
	3% EtOH	580	1,900	1,700	6.2
37°C	No EtOH	580	580	480	1.8
	3% EtOH	300	540	420	3.2

[a]Samples from an exponential culture of strain TB4 were pulse-labeled with 10 μCi [1-^{14}C] acetate (61 mCi/mmole) at various temperatures in the presence and absence of 3% ethanol. Fatty acids from purified phospholipids were separated by argentation chromatography.

[b]UFA, unsaturated fatty acid; SFA, saturated fatty acid

primarily the result of an absolute decrease in esterified saturated fatty acids. Unlike ethanol addition, a shift to lower temperature was also accompanied by an absolute increase in esterified unsaturated fatty acids. A shift to higher temperature caused an inhibition of acetate incorporation into both saturated and unsaturated fatty acids which was further inhibited by the inclusion of ethanol.

The stimulation of lipid synthesis by a shift to lower temperature and its inhibition by a shift to higher temperature are difficult to explain. These changes may be related to changes in the levels of guanosine-5'-diphosphate-3'-diphosphate (ppGpp). High levels of ppGpp inhibit both phospholipid synthesis (Merlie and Pizer, 1973) and fatty acid synthesis (Nunn and Cronan, 1976). Recent studies by Gallant et al. (1977) have shown that E. coli rapidly accumulates this nucleotide upon shifting to a higher temperature.

Effect of Fatty Acid Degradation (fadE) on Ethanol-Induced Changes in Fatty Acid Composition. Since fatty acid synthesis is tightly coupled to phospholipid synthesis, the changes observed in UFA/SFA ratios could be due to the direct action of ethanol on the availability of fatty acids, or their utilization by the acyltransferase system, or both. E. coli possesses a multi-enzyme complex capable of fatty acid degradation (Binstock et al., 1977). This enzyme complex may partially determine the fatty acid composition of membrane lipids by altering the relative amounts of fatty acid species available for incorporation into phospholipids. Thus, the ethanol-induced reduction in palmitic acid previously reported in E. coli (Ingram, 1976)

could result from a preferential degradation of endogenously synthesized palmitic acid. To determine if this was indeed the case, the effects of ethanol on strain CSH2, proficient in fatty acid degradation (*fadE*$^+$), and strain TB4, defective in fatty acid degradation (*fadE*$^-$), were compared (Table 4). In both strains the proportion of palmitic acid (16:0) was reduced by 10%, with an equal increase in the proportion of *cis*-vaccenic acid during growth in the presence of ethanol. Neither strain showed a significant change in the level of palmitoleic acid (16:1). These results indicate that preferential fatty acid degradation is not responsible for ethanol-induced changes in fatty acid composition.

Effect of Ethanol on Free Fatty Acid Synthesis. Although fatty acid degradation is not essential for ethanol-induced fatty acid changes (Table 4), the availability of fatty acids for phospholipid synthesis could also be modified by a direct effect of ethanol on fatty acid synthesis. In *E. coli*, fatty acid synthesis is normally tightly coupled with phospholipid synthesis (Cronan, 1974) and prevents an evaluation of the effect of ethanol on either of these systems independent of the other. In order to determine if ethanol directly affects fatty acid synthesis, we have used two mutants in which fatty acid synthesis can be uncoupled from phospholipid synthesis *in vivo* by glycerol-3-phosphate starvation. The removal of exogenous glycerol-3-phosphate from growing cells of strains LW1 and LW3 causes an inhibition of phospholipid synthesis and an accumulation of free (non-esterified) fatty acids (Cronan *et al.*, 1975). These are easily labeled by pulsing

Table 4. Effect of fatty acid degradation of ethanol-induced changes in fatty acid composition[a]

Strain	Conditions	Fatty Acid Composition (%)				UFA/SFA Ratio[b]
		14:0	16:0	16:1(Δ17)	cis-Δ^{11}-18:1	
CSH2 (*fadE*$^+$)	No EtOH	3.1(0.5)	32.9(0.2)	36.6(0.8)	26.8(1.3)	1.7
	3% EtOH	1.9(0.6)	21.9(0.5)	37.1(1.1)	38.1(0.6)	3.0
TB4 (*fadE*$^-$)	No EtOH	3.3(0.5)	31.5(0.4)	35.5(0.5)	28.5(0.9)	1.8
	3% EtOH	1.9(0.5)	20.9(1.2)	37.4(0.8)	39.1(1.4)	3.2

[a]Cells were grown in the presence and absence of alcohol, harvested and analyzed by gas chromatography. These results represent an average of four separate experiments and are expressed as a percentage of total fatty acids with the standard deviation in parentheses.

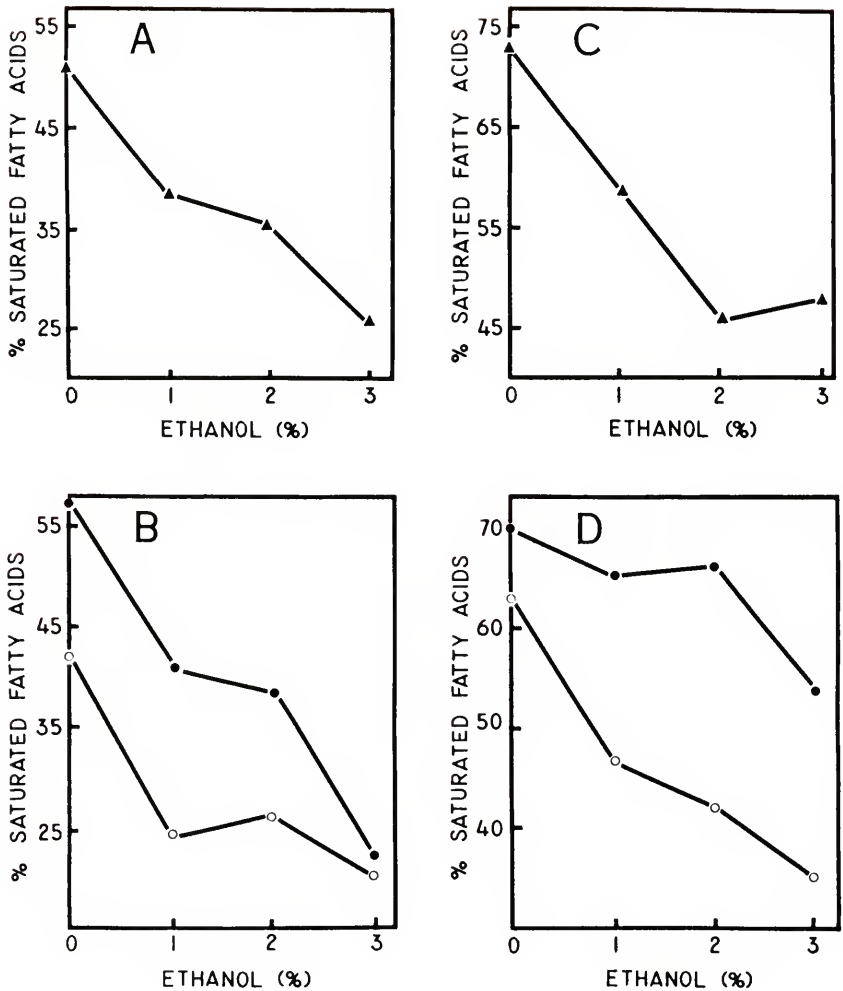
[b]UFA, unsaturated fatty acid; SFA, saturated fatty acid.

the cells with [^{14}C] acetate during starvation. Subsequent analysis of the free fatty acids provides an _in vivo_ method for examining the regulation of fatty acid synthesis, _per se_, independent of the acyltransferase system.

The addition of ethanol to strains LW1 and LW3 altered the proportions of saturated and unsaturated free fatty acids which accumulated (Figure 2A and C). Although the synthesis of both saturated as well as unsaturated fatty acids was inhibited by ethanol ([^{14}C] acetate incorporation), 3% ethanol caused only a 23% decrease in the synthesis of unsaturated fatty acids while causing a 73% reduction in the synthesis of saturated fatty acids. Thus the ethanol-induced increase in the UFA/SFA ratio during the free fatty acid synthesis results from the preferential inhibition of saturated fatty acid synthesis.

In order to determine whether ethanol affects the incorporation of fatty acids into newly synthesized phospholipids in strain LW1 and LW3, we also examined the fatty acid composition of the phospholipids. In the absence of exogenous glycerol-3-phosphate, a small amount of residual phospholipid synthesis continues in these strains (Cronan _et al._, 1975). The ratio of unsaturated to saturated fatty acids either in these residual phospholipids or in the phospholipids of cells supplemented with glycerol-3-phosphate can be compared with the ratios in the accumulated free fatty acids to determine the effect of ethanol on the selectivity of the phospholipid synthesizing enzymes. Figure 2 (B and D) demonstrates that phospholipids extracted from glycerol-3-phosphate supplemented cells as well as from glycerol-3-

Figure 2. Effect of ethanol on free fatty acid synthesis and total lipid synthesis in strains LW1 and LW3. Exponential cultures grown with glycerol-3-phosphate supplementation were filtered, washed and resuspended in fresh media minus glycerol-3-phosphate. Cultures were split and one-half of each culture was supplemented with glycerol-3-phosphate. Growth was continued for 3 hours at which time one-milliliter samples were removed from the cultures and added to tubes containing 10 μCi of [1-^{14}C] acetate (61 mCi/mmole) and various concentrations of ethanol. After ten minutes the reaction was stopped by the addition of chloroform-methanol (1:2). Lipids were extracted and analyzed by argentation chromatography as described in the "Experimental Procedure" section. A, Free fatty acid synthesis in strain LW3 starved for glycerol-3-phosphate. B, Incorporation of fatty acids into phospholipids of strain LW3 starved for glycerol-3-phosphate (●) and supplemented with glycerol-3-phosphate (○). C, Free fatty acid synthesis in strain LW1 starved for glycerol-3-phosphate. D, Incorporation of fatty acids into phospholipids of strain LW1 starved for glycerol-3-phosphate (●) and supplemented with glycerol-3-phosphate (○).


A
% SATURATED FATTY ACIDS
55
45
35
25
0
1
2
3
ETHANOL (%)
C
% SATURATED FATTY ACIDS
75
65
55
45
0
1
2
3
ETHANOL (%)
B
% SATURATED FATTY ACIDS
55
45
35
25
0
1
2
3
ETHANOL (%)
D
% SATURATED FATTY ACIDS
70
60
50
40
0
1
2
3
ETHANOL (%)

phosphate starved cells contained reduced levels of saturated fatty acids as a result of ethanol addition. Further, the dose-related reductions of saturated fatty acids incorporated into phospholipids are very similar to the dose-dependent inhibition of de novo saturated fatty acid biosynthesis. This suggests that the ethanol-induced changes in the fatty acid composition of newly synthesized phospholipids arise prior to phospholipid synthesis and that ethanol may have little effect upon the selectivity of fatty acid utilization for phospholipids. Our results with strains LW1 and LW3 demonstrate that ethanol acts directly at the level of fatty acid synthesis, preferentially inhibiting the synthesis of saturated fatty acids.

Effect of Ethanol on the Fatty Acid Composition of an Unsaturated Fatty Acid Auxotroph. Our results thus far demonstrate that the addition of ethanol causes a reduction in the proportion of saturated fatty acids available for incorporation into phospholipids. This reduction could reflect an increased availability of unsaturated fatty acids for phospholipid synthesis resulting from a stimulation of unsaturated fatty acid synthesis by ethanol. To test this, we used an unsaturated fatty acid auxotroph, strain K1060, which derives all of its esterified unsaturated fatty acids from exogenous supplements (Overath et al., 1970).

Strain K1060 was grown in the presence and absence of ethanol with several unsaturated fatty acid supplements (Table 5). When supplemented with either palmitoleic acid (16:1) or oleic acid (cis-Δ^9-18:1), the addition of ethanol resulted in an increase in the

Table 5. Effect of ethanol on the fatty acid composition of an unsaturated fatty acid auxotroph, strain K1060[a]

Unsaturated Supplement	Conditions	Fatty Acid Composition (%)					UFA/SFA Ratio[b]
		14:0	16:0	16:1	cis-Δ^9-18:1	20:2	
16:1	No EtOH	5.7	44.1	48.9	----	----	0.96
	3% EtOH	4.3	38.4	56.1	----	----	1.28
cis-Δ^9-18:1	No EtOH	6.1	31.6	----	62.3	----	1.65
	3% EtOH	3.5	27.0	----	69.5	----	2.28
20:2	No EtOH	14.4	46.0	----	----	39.6	0.66
	3% EtOH	9.2	40.8	----	----	49.9	1.00

[a]Cells were grown in the presence and absence of 3% ethanol with different fatty acid supplements. Phospholipids were separated by thin layer chromatography prior to fatty acid analysis by gas chromatography. Results are expressed as a percentage of total fatty acids.

[b]UFA, unsaturated fatty acids; SFA, saturated fatty acids.

proportion of esterified unsaturated fatty acids. Further, this ethanol-induced change was not dependent upon specific unsaturated acyl chains. E. coli does not normally synthesize di-unsaturated fatty acids or fatty acids of chain lengths greater than 18 carbons. However, strain K1060 supplemented with eicosadienoic acid (20:2) still underwent significant changes in the proportion of unsaturated fatty acids in response to ethanol (Table 5). These results demonstrate that ethanol does not act by stimulating unsaturated fatty acid synthesis, and that the regulation of unsaturated fatty acid synthesis is not essential for the ethanol response.

Effect of Ethanol on the Incorporation of Exogenous Fatty Acids into Phospholipids in the Absence of de novo Fatty Acid Synthesis. We have shown that ethanol increases the UFA/SFA ratio produced during de novo fatty acid synthesis by preferentially inhibiting the synthesis of saturated fatty acids. The dose-dependent shift in this ratio is almost identical to that observed following incorporation into phospholipids, suggesting that the acyltransferase system may not be affected directly by ethanol (Figure 2). This differs from temperature adaptation of fatty acid composition, which has been shown by other workers to be regulated by both fatty acid synthesis (Cronan, 1975; Okuyama et al., 1977) and the selectivity of the acyltransferase system (Esfahani et al., 1969; Sinensky, 1971). However, it is possible that in vivo the selectivity of the acyltransferase enzymes is only a secondary means of regulating fatty acid composition. Cells which can regulate their fatty acid composition at the level of fatty acid synthesis may not require or exhibit additional changes in

selectivity of incorporation of fatty acids into phospholipids (Okuyama et al., 1977). Indeed, the similarity between synthesized fatty acids and phospholipid composition in E. coli in response to growth temperature (Cronan, 1975) would seem to support this theory. Thus the effect of ethanol on fatty acid synthesis may obscure any effect of the alcohol on the acyltransferase enzymes. To test this possibility, the effect of ethanol on the incorporation of exogenous fatty acids into phospholipids in vivo was examined under conditions which minimize de novo fatty acid synthesis. Strains L8 and L48 synthesize fatty acids at one-fourth the normal rate when shifted to 37°C, and therefore require supplementation with both saturated and unsaturated fatty acids for phospholipid synthesis (Harder et al., 1972).

The results in Table 6 show that ethanol caused a slight inhibition of the incorporation of exogenous 16:0 and cis-Δ^9-18:1 fatty acids into phospholipids, but did not alter the ratio of their incorporation. These results demonstrate that ethanol has little direct effect upon the selectivity of the acyltransferase enzymes.

Effect of Exogenous Fatty Acids on Ethanol-Induced Changes in Fatty Acid Composition. Our results have shown that ethanol preferentially inhibits the synthesis of saturated fatty acids. The ethanol-induced reduction in the proportion of saturated fatty acids in cellular phospholipids could simply result from a reduced availability. To examine this possibility, we have determined the fatty acid composition of phospholipids from strain TB4 grown with a series of concentrations of 16:0 in the presence and absence of ethanol.

Table 6. Effect of ethanol on the incorporation of fatty acids into phospholipids[a]

	Fatty Acids Incorporated[b]					
	No EtOH			3% EtOH		
Strain	Total	Saturated	Unsaturated	Total	Saturated	Unsaturated
L8	298	216	82	266	193	73
L48	283	204	79	225	161	64

[a]Cells were grown with palmitic acid and oleic acid supplements at 37°C, to a density of 10^8 cells/ml. The cells were harvested by centrifugation and resuspended in one-half volume of fresh media. Samples (1 ml) were removed and added to tubes containing 1 ml of media, 50 µCi of [9,10(n)-^{3}H] oleic acid (2.2 Ci/mmole) and 1.3 µCi of [1-^{14}C] palmitic acid (56 mCi/mmole) in the presence and absence of ethanol. Phospholipids were separated by thin layer chromatography and analyzed by liquid scintillation counting.

[b]picamoles of fatty acid/sample.

Supplementation with exogenous palmitic acid increased the proportion of esterified 16:0 with decreases in both cis-Δ^{11}-18:1 and 16:1 fatty acids (Table 7). A similar pleiotropic effect of 16:0 was previously reported by Silbert et al. (1973a). The increase in abundance of esterified 16:0 was nearly constant at all levels of supplementation. Further, supplementation with palmitic acid did not prevent the ethanol-induced decrease in esterified 16:0. This decrease in esterified palmitic acid was compensated for primarily by an increase in 16:1 fatty acid rather than cis-Δ^{11}-18:1, in contrast to the previous experiments without supplements.

The results in Table 7 would seem to suggest that the ethanol-induced reduction in esterified 16:0 is not due solely to a limitation of 16:0 available to the cell. However, it is not clear to what extent the activated form of 16:0 is available to the acyltransferase system. Thus the apparent failure of excess 16:0 to eliminate the ethanol-induced reduction in esterified palmitic acid could result from a limited ability to transport or activate exogenous 16:0, consistent with the lack of a dose-dependent response to 16:0 supplement. Alternatively, ethanol could directly inhibit the transport or activation of exogenously supplied 16:0. Either of these coupled with a high affinity of the acyltransferase enzymes for 16:0 would lead to changes in fatty acid composition which still predominantly reflect the biosynthetic production of 16:0. To investigate these possibilities, we have examined the effect of ethanol on the contribution of de novo biosynthesis and of exogenously supplied 16:0 to

Table 7. Effect of exogenous 16:0 on ethanol-induced changes in fatty acid composition in strain TB4

Supplement (mg/L)	Fatty Acid Composition (%)							
	No EtOH				3% EtOH			
	14:0	16:0	16:1(Δ17)	cis-Δ^{11}-18:1	14:0	16:0	16:1(Δ17)	cis-Δ^{11}-18:1
0	3.3	28.0	38.1	30.1	1.7	20.8	35.4	41.9
5	4.3	54.6	32.3	7.9	1.0	45.0	41.6	11.5
10	2.9	58.1	30.0	8.1	0.9	47.6	39.3	11.9
50	5.9	62.7	23.9	6.9	1.0	51.5	33.4	13.5
100	2.3	51.8	34.7	10.6	1.1	45.5	37.5	15.9

[a]Cells were grown in the presence and absence of ethanol with various concentrations of palmitic acid (supplement), harvested at a density of 2 x 10^8 cells/ml and analyzed by gas chromatography. Results are expressed as a percentage of total fatty acids.

esterified lipids in both pulse-label experiments and also in bulk lipid composition.

Double label experiments were performed with strain TB4 using [^{14}C] acetate to label esterified palmitic acid derived from biosynthesis and [^{3}H] palmitic acid to label that derived from supplements (Table 8). In strain TB4, the incorporation of exogenously supplied 16:0 [^{3}H] was slightly stimulated by the addition of ethanol while the incorporation from biosynthesis [^{14}C] was strongly inhibited. These results confirm that ethanol inhibits saturated fatty acid synthesis and demonstrate that ethanol does not decrease the availability of exogenously supplied 16:0 to the acyltransferase enzymes.

The results of the pulse-label experiments were further confirmed in a second organism, strain K1060, using exogenously supplied 17:0 and cis-Δ^{9}-18:1 fatty acids. Heptadecanoic acid (17:0) is used by *E. coli* as a 16:0 equivalent (Silbert *et al.*, 1973b; Ingram *et al.*, 1977). In the absence of a direct effect of ethanol on the utilization of exogenous fatty acids for esterification, the ratio of esterified (14:0 + 16:0)/17:0 provides a measure of saturated fatty acid biosynthesis. These fatty acids are easily resolved by gas chromatography. As observed in pulse-label experiments, the utilization of saturated fatty acids derived from biosynthesis (16:0 + 14:0) and of that derived from exogenous supplements (17:0) did not follow the same trends in response to ethanol addition (Table 9). The addition of ethanol resulted in a dramatic increase in the proportion of exogenously supplied saturated fatty acid (17:0)

Table 8. Effect of ethanol on the incorporation of exogenous 16:0 [^{3}H] and endogenous 16:0 [^{14}C] into the phospholipids of strain TB4[a]

	Palmitic Acid Incorporated into Phospholipids (cpm)		
Conditions	Endogenous [^{14}C]	Exogenous [^{3}H]	Ratio[b]
No EtOH	2,500	18,200	7.3
3% EtOH	310	20,800	67.1

[a]Samples from an exponential culture were pulsed for 10 minutes with 10 µCi of [1-^{14}C] acetate (61 mCi/mmole) and 10 µCi/ml of [9,10(n)-^{3}H] palmitic acid (442.5 mCi/mmole). Lipids were extracted and the phospholipids separated using thin layer chromatography.

[b]Ratio of the [^{3}H] cpm to [^{14}C] cpm.

Table 9. Effect of ethanol on the incorporation of 17:0 into the phospholipids of an unsaturated fatty acid auxotroph, strain K1060[a]

	Fatty Acid Composition (%)				
Conditions	14:0	16:0	17:0	cis-Δ^9-18:1(Δ19)	Ratio (14:0+16:0)/17:0
No Ethanol	7.0	40.7	25.4	26.9	1.9
3% Ethanol	3.4	25.8	41.2	29.6	0.7

[a]Cells were grown with both 17:0 and 18:1 fatty acids in the presence and absence of ethanol. Phospholipids were purified by thin layer chromatography and analyzed by gas chromatography. Results are expressed as a percentage of total fatty acids.

and a simultaneous decrease in the proportion of saturated fatty acids derived from biosynthesis (16:0 + 14:0). Further, the ethanol-induced increase in the proportion of unsaturated fatty acid (18:1) was also reduced in strain K1060 as compared to strain TB4 grown with a saturated fatty acid supplement (Table 7). This may be due to the more efficient transport or utilization of exogenous fatty acids by strain K1060. Spontaneous secondary mutations appear to be selected for in fatty acid auxotrophs (Fox et al., 1970). Thus the exogenous supply of saturated fatty acids to this mutant partially compensates for the direct inhibition of saturated fatty acid synthesis by ethanol. These results provide further evidence that the ethanol-induced changes in fatty acid composition do not result from a direct effect of ethanol on the selectivity of the acyltransferase system in vivo.

Effect of Ethanol on Fatty Acid Synthesis in Vitro. If ethanol is acting at the level of fatty acid synthesis in E. coli, it should be possible to demonstrate an effect on the fatty acids synthesized in vitro. A crude preparation of fatty acid synthesizing enzymes was prepared from E. coli, and the fatty acids synthesized in vitro were separated by argentation chromatography. The effect of ethanol on fatty acid synthesis in vitro is demonstrated in Table 10.

Ethanol preferentially inhibited saturated fatty acid synthesis in vitro in a manner similar to the preferential inhibition observed in vivo. The addition of divalent cations (5 mM) could not prevent this inhibition (Table 10), suggesting that ethanol does not disrupt an essential ion-protein interaction. Therefore, to determine the

Table 10. Effect of ethanol on the fatty acids synthesized *in vitro* by a crude fatty acid synthetase[a]

Experiment	Fatty Acids Synthesized (cpm)			
	SFA[b]	UFA	PFA	UFA/SFA
1 (Na^+K^+)				
No EtOH	266,389	351,847	49,406	1.32
3% EtOH	182,426	396,106	58,682	2.17
2 ($+Mg^{++}$)				
No EtOH	313,563	434,349	51,194	1.38
3% EtOH	181,812	424,006	57,729	2.33
3 ($+Ca^{++}$)				
No EtOH	194,379	299,044	60,363	1.54
3% EtOH	85,067	254,799	61,393	2.99

[a]Fatty acids were synthesized *in vitro* by a crude enzyme preparation. Incubation was carried out at 37°C for 45 minutes in the presence and absence of ethanol. Fatty acids were transesterified and separated by argentation chromatography.

[b]SFA, saturated fatty acids; UFA, unsaturated fatty acids; PFA, polar fatty acids.

mechanism by which ethanol affects fatty acid synthesis, the crude enzymes were taken through two additional steps of purification.

Crude enzymes were centrifuged at 100,000 x g for two hours to remove most of the membrane fragments, and the resulting enzyme is referred to as the 100 Kg enzyme preparation. An aliquot of the 100 Kg preparation was further purified by ammonium sulfate fractionation as described by Lennarz et al. (1962), and this enzyme represents the $(NH_4)_2SO_4$ preparation. The three different preparations were then assayed in the presence and absence of ethanol, and the products were analyzed by argentation chromatography.

The results in Table 11 demonstrate that all three enzyme preparations were markedly affected by the presence of ethanol. In each case, the addition of ethanol resulted in a preferential inhibition of saturated fatty acid synthesis, resulting in an increased UFA/SFA ratio. Thus ethanol seems to be exerting its effect directly at the level of the fatty acid synthesizing enzymes.

It seemed possible that ethanol could be affecting saturated fatty acid synthesis in a manner which would lead to the arrest of further saturated fatty acid synthesis without affecting unsaturated fatty acid synthesis. To test this, ammonium sulfate enzyme preparations were incubated for increasing periods of time in the presence and absence of ethanol. The synthesized fatty acids were separated by argentation chromatography and the amounts of saturated and unsaturated fatty acids determined.

Figure 3 demonstrates the effects of ethanol on fatty acid synthesis in vitro. Both saturated fatty acid synthesis as well as

Table 11. Effect of purification of fatty acid synthetase on ethanol-induced fatty acid changes *in vitro*[a]

Enzyme	Conditions	Fatty Acids Synthesized (cpm)			
		SFA[b]	UFA	PFA	UFA/SFA
Crude	-EtOH	42,463	106,240	17,651	2.50
	3% EtOH	24,983	84,586	17,247	3.39
100 Kg	-EtOH	69,341	112,312	13,477	1.62
	3% EtOH	48,904	124,379	19,061	2.54
$(NH_4)_2SO_4$	-EtOH	76,396	108,604	11,391	1.42
	3% EtOH	51,779	116,187	12,449	2.24

[a] Fatty Acids were synthesized *in vitro* by enzymes of different levels of purification. Incubation was carried out at 37°C for 45 minutes in the presence and absence of ethanol. Fatty acids were transesterified and separated by argentation chromatography.

[b] SFA, saturated fatty acids; UFA, unsaturated fatty acids; PFA, polar fatty acids.

unsaturated fatty acid synthesis were inhibited by ethanol, but the inhibition of saturated fatty acid synthesis was greater. In the presence of 4% ethanol, the rate of unsaturated fatty acid synthesis was decreased by 30%, while the rate of saturated fatty acid synthesis was inhibited by 48%. In a duplicate experiment, unsaturated fatty acid synthesis was not inhibited, while saturated fatty acid synthesis was inhibited by 30%. Thus saturated fatty acid synthesis is not totally arrested, but rather it occurs at a slower rate than unsaturated fatty acid synthesis.

In the presence of ethanol, short-chained saturated fatty acids (decanoic and dodecanoic) may be synthesized, but their subsequent elongation to myristic or palmitic acids could be inhibited. To examine this possibility, fatty acids were synthesized *in vitro* in the presence and absence of 4% ethanol. The products were separated into saturated, unsaturated and polar fatty acids by argentation chromatography (Figure 4). Regions corresponding to these fatty acids were eluted with pentane and rechromatographed. Saturated and unsaturated fatty acids were separated on the basis of chain length by reversed phase chromatography; polar fatty acids were rechromatographed on argentation thin layer plates. The results of this experiment are shown in Table 12.

In agreement with a previous report (Lennarz *et al.*, 1962), the fatty acids synthesized *in vitro* were found to be of greater chain lengths than fatty acids synthesized *in vivo*. This is thought to result from an absence of phospholipid synthesis, which would normally utilize the palmitic, palmitoleic and vaccenic acids before they can

Figure 3. Effect of ethanol on the rates of synthesis of saturated and unsaturated fatty acids *in vitro*. Fatty acid synthetase enzymes purified by ammonium sulfate fractionation were added to the standard reaction mixture. The assay mixtures were incubated at 37°C for various periods of time in the presence and absence of 4% ethanol. Labeled fatty acids were extracted and analyzed by argentation chromatography. Symbols: (▲), unsaturated fatty acid synthesis in the absence of ethanol; (△), unsaturated fatty acid synthesis in the presence of ethanol; (■), saturated fatty acid synthesis in the absence of ethanol; (□), saturated fatty acid synthesis in the presence of ethanol.

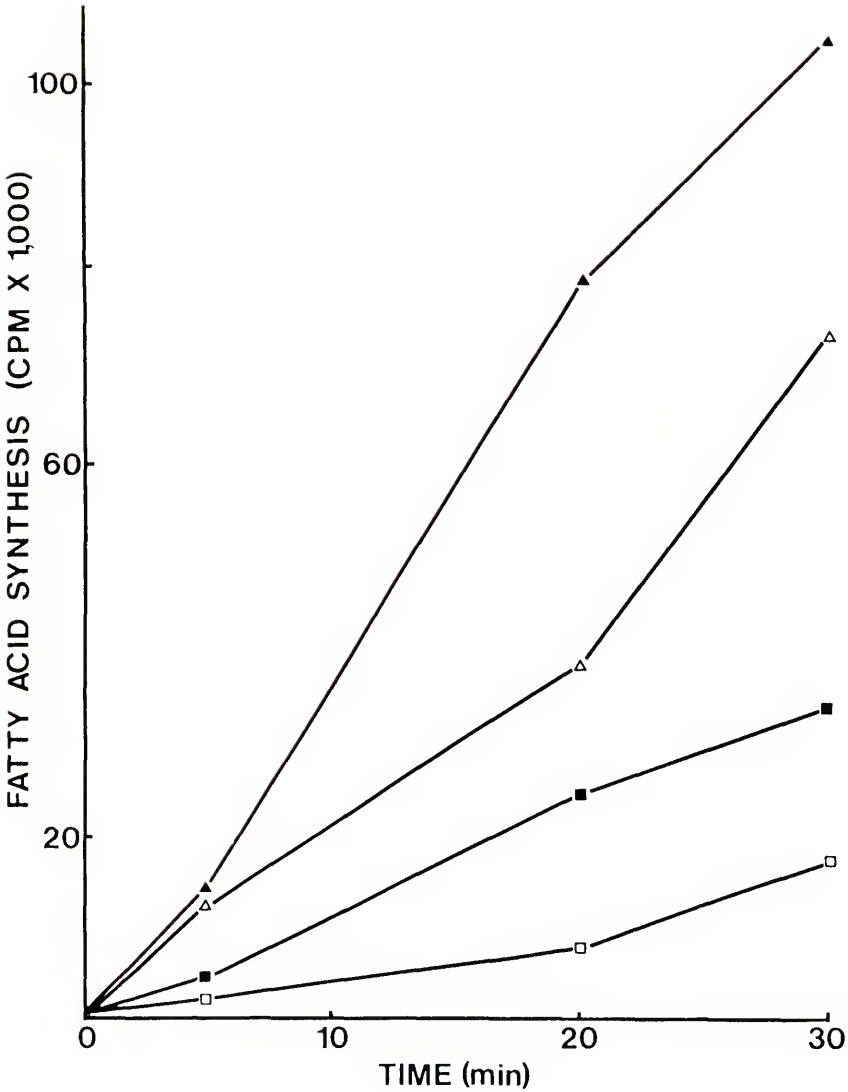

FATTY ACID SYNTHESIS (CPM X 1,000)
100
60
20
0
10
20
30
TIME (min)

Figure 4. Argentation chromatography of fatty acid products synthesized *in vitro*. Fatty acid synthesis enzymes, purified by ammonium sulfate fractionation, were used to synthesize fatty acids *in vitro*. Assay mixtures were incubated for 30 minutes in the presence and absence of 4% ethanol. Labeled fatty acids were extracted and analyzed by argentation chromatography. Regions corresponding to the radioactive fatty acids were located by autoradiography. Designated regions represent: SFA, Saturated fatty acids; UFA, Unsaturated fatty acids; PFA, Polar fatty acids.

No EtOH
4% EtOH
SFA
cis-Δ15-22:1
cis-Δ13-20:1
UFA
cis-Δ11-18:1
cis-Δ9-16:1
A
B
PFA
C

be further elongated (Cronan et al., 1975). In the absence of ethanol, large amounts of stearic, arachidic and eicosenoic fatty acids were synthesized due to the continued elongation of palmitic and vaccenic acids. In the presence of 4% ethanol there is a shift to shorter fatty acids, as demonstrated by the marked reduction observed in arachidic and eicosenoic acids. However, the high levels of palmitic and stearic acids synthesized as well as the absence of any shorter chain intermediates (lauric or myristic acids) demonstrate that ethanol is not preventing the complete elongation of saturated fatty acids. Since ethanol also causes a reduction in the chain lengths of the unsaturated fatty acids observed in vitro (Table 12), the observed reduction in chain lengths may result from an interaction of ethanol with the condensing enzymes responsible for elongating the fatty acid intermediates.

Despite the separation of the polar fatty acids (β-hydroxy intermediates) into three distinct regions by argentation chromatography, we were unable to identify these different polar acids. Although there was an increase in fraction C in the presence of ethanol (Table 12), we were not able to demonstrate an accumulation of any specific intermediate. Thus the inhibition by ethanol of a specific enzyme cannot be demonstrated by the accumulation of a specific enzyme's precursor. These results suggest that the ethanol-induced reduction in saturated fatty acid synthesis is due to a greater inhibition of the rate of saturated fatty acid synthesis as compared to the rate of unsaturated fatty acid synthesis.

Table 12. Effect of ethanol on the chain lengths of the fatty acid products synthesized *in vitro*[a]

Fatty Acids	Rf	No EtOH		4% EtOH	
		cpm	%	cpm	%
Saturated		28,192	100	4,204	100
Palmitic (16:0)	.53	7,136	25.3	1,767	42.0
Stearic (18:0)	.43	13,418	47.6	1,966	46.8
Arachidic (20:0)	.33	6,125	21.7	345	8.2
Behenic (22:0)	.24	1,513	5.4	126	3.0
Unsaturated		107,292	100	68,115	100
<18:1	>.67	12,195	11.4	8,669	12.7
cis-Vaccenic (18:1)	.59	64,045	59.7	46,595	68.4
cis-13-Eicosenoic (20:1)	.48	28,255	26.3	11,790	17.3
cis-15-Docosenoic (22:1)	.38	2,797	2.6	1,061	1.6
Polar		9,187	100	17,353	100
A (Unidentified)	.25	2,928	31.9	3,619	20.9
B (Unidentified)	.18	1,166	12.7	2,497	14.4
C (Unidentified)	<.16	5,093	55.4	11,237	64.8

[a]Assay mixtures were incubated at 37°C for 30 minutes in the presence and absence of 4% ethanol. Fatty acids were extracted and separated by argentation chromatography. Regions corresponding to saturated, unsaturated and polar fatty acids were eluted with pentane and rechromatographed either by reversed phase chromatography (saturates and unsaturates) or by argentation chromatography (polar fatty acids).

The fatty acid changes induced by alcohols are similar to the fatty acid changes induced by temperature shifts (Marr and Ingraham, 1962). Recent evidence by Cronan (1975) suggests that *in vivo*, fatty acid synthesis is one site of thermal control of phospholipid fatty acid composition. Therefore, it seemed appropriate to investigate the effects of incubation temperature on fatty acid synthesis *in vitro*. Enzymes purified by ammonium sulfate fractionation were used to synthesize fatty acids at three incubation temperatures, and the results of this experiment are given in Table 13.

Increasing incubation temperature resulted in a corresponding increase in the relative amounts of saturated fatty acids synthesized *in vitro*. These results are in agreement with those reported by Okuyama *et al*. (1977), and provide evidence that temperature adaptation of fatty acid composition does not depend solely upon interactions with the cell membrane *per se*. The effect of temperature is also exerted directly upon the fatty acid synthetase enzymes. These results with temperature suggest that the fatty acid changes induced by temperature shifts and the fatty acid changes induced by ethanol may be mediated through a common mechanism.

Table 13. Effect of incubation temperature on the fatty acid products synthesized *in vitro*[a]

	SFA[b]		UFA		PFA		
Temperature	cpm	(%)[c]	cpm	(%)	cpm	(%)	UFA/SFA
32°C	14,917	(19.3)	52,137	(67.5)	10,246	(13.2)	3.50
37°C	15,229	(24.3)	38,881	(62.2)	8,443	(13.5)	2.55
42°C	30,668	(36.6)	43,939	(52.4)	9,199	(11.0)	1.43

[a]Fatty acid synthetase enzymes were purified by ammonium sulfate fractionation. Enzymes were added to assay mixtures and incubated at either 32°C, 37°C or 42°C for 30 minutes. Fatty acids were extracted and separated by argentation chromatography.

[b]SFA, saturated fatty acids; UFA, unsaturated fatty acids; PFA, polar fatty acids.

[c]Expressed as percent of total fatty acids synthesized at the indicated temperature.

DISCUSSION

It has previously been shown that the addition of ethanol to E. coli caused a reduction in the proportion of palmitic acid (16:0) esterified into membrane lipids (Ingram, 1976). This decrease in palmitic acid was subsequently found to occur in both phosphatidyl glycerol and phosphatidyl ethanolamine (Ingram, 1977a), the major phospholipids present in E. coli (Cronan and Vagelos, 1972). The experiments described in this dissertation represent an extension of these previous reports, and were designed to determine the mechanisms by which ethanol induced fatty acid changes. Fatty acid degradation, fatty acid synthesis, and fatty acid utilization (incorporation into phospholipids) were considered as possible sites for the interaction with ethanol. The results presented in this dissertation provide evidence that these changes in fatty acid composition result primarily from the preferential inhibition of saturated fatty acid synthesis by ethanol.

Several observations support this conclusion: (1) Mutants deficient in fatty acid degradation showed an ethanol-induced change in their fatty acid composition identical to that observed in wild-type cells (Table 4). Thus the addition of ethanol does not lead to reduced incorporation of palmitic acid into phospholipids as a result of preferential degradation of the saturated fatty acids. (2) The immediacy of the ethanol-induced changes in the fatty acid

composition of newly synthesized phospholipids, as well as their equally rapid reversal upon ethanol removal, suggests that ethanol is acting directly at the level of lipid synthesis (Table 2). (3) In an unsaturated fatty acid auxotroph, the ratio of unsaturated to saturated fatty acids incorporated into phospholipids was elevated in response to ethanol. This elevation occurred regardless of the unsaturated fatty acid supplement provided (Table 5). Thus the increase in the proportion of unsaturated fatty acids did not result from a stimulation of their synthesis by ethanol. (4) When fatty acid synthesis was uncoupled from phospholipid synthesis, ethanol caused a dose-related reduction in the proportion of saturated fatty acids synthesized (Figures 2A and C). This indicates that ethanol is acting directly at the level of fatty acid synthesis. In addition, the similarity between the ratios of free fatty acids and esterified fatty acids suggested that the effect of ethanol on fatty acid synthesis is responsible for most, if not all, of the observed reduction in esterified palmitic acid. (5) In the absence of endogenous fatty acid synthesis, ethanol did not alter the ratios of fatty acids incorporated into phospholipids (Table 6). Unlike temperature adaptation, adaptation to ethanol is not mediated by altering the selectivity of the acyltransferase enzymes. These in vivo experiments demonstrate that the endogenous synthesis of saturated fatty acids is essential for ethanol-induced fatty acid changes to occur. The ability to eliminate several lipid synthesizing and degradative processes without eradicating the ethanol-response is in agreement with our conclusion that ethanol preferentially inhibits saturated fatty acid synthesis.

This conclusion is further supported by the results of our experiments with ethanol and the fatty acid synthetase enzymes *in vitro*. In crude, cell-free extracts, the addition of ethanol resulted in a preferential inhibition of saturated fatty acid synthesis in a fashion analogous to the effects *in vivo*. This effect could not be eliminated by the addition of excess magnesium and calcium to the assay mixture (Table 10). The crude enzymes were taken through two additional steps of purification to try and determine the level of the ethanol interaction. The removal of most of the membrane lipids by ultracentrifugation did not prevent the ethanol-induced inhibition of saturated fatty acid synthesis (Table 11). Thus the effects of ethanol in the cell membrane, *per se*, are not involved in the ethanol-induced changes in fatty acid composition. However, since some proteins are surrounded by a lipid annulus that cannot be removed by simple ultracentrifugation (Fourcans and Jain, 1974), the possibility of an ethanol effect at the level of a lipid-protein association cannot be excluded.

The membrane-free preparation was further purified by ammonium sulfate fractionation to remove excess protein. The addition of ethanol to a reaction mixture catalyzed by this preparation also resulted in the preferential inhibition of saturated fatty acid synthesis (Table 11). Based upon this experiement, we conclude that ethanol is acting directly at the level of the fatty acid synthetase enzymes.

The specific enzyme(s) affected by ethanol are not known. *In vitro* experiments aimed at elucidating the specific enzyme(s)

demonstrated that ethanol does not totally arrest saturated fatty acid synthesis, nor does ethanol prevent the synthesis of long chain saturated fatty acids. Rather, ethanol seems to be inhibiting the rate of saturated fatty acid synthesis to a greater degree than it inhibits the rate of unsaturated fatty acid synthesis, leading to the observed increase in the UFA/SFA ratio. This effect could be produced by an interaction of ethanol with at least two enzymes: β-hydroydecanoyl dehydrase or β-ketoacyl-acyl ACP synthetase.

β-Hydroydecanoyl dehydrase catalyzes a double-bond formation in the ten carbon intermediate in fatty acid synthesis in E. coli. This enzyme is capable of forming an α,β trans double bond, a β,γ cis double bond, or an isomerization between the two forms (Kass et al., 1967). If a trans double bond is produced, the α,β trans decenoyl ACP derivative will be reduced to decanoyl ACP followed by a subsequent elongation to palmitic acid (16:0). However, when a cis double bond is introduced it is not subsequently reduced, and the β,γ cis decenoyl ACP is elongated to form palmitoleoyl ACP (16:1) and cis vaccenoyl ACP (18:1). Therefore, if ethanol altered the relative rates at which the enzyme introduced cis or trans double bonds, the final proportions of fatty acids synthesized could be modified to include less saturated fatty acids. Unfortunately, a reduction in trans double bond formation might be expected to yield an increase in cis bond formation, and hence an increase in unsaturated fatty acids. Since the addition of ethanol also inhibited total fatty acid synthesis, it could not be determined if such an increase in unsaturated fatty acid synthesis was actually occurring.

An attractive alternative for the site of ethanol interaction is the condensing enzyme responsible for saturated fatty acid synthesis in E. coli. The presence of two condensing enzymes have recently been demonstrated in E. coli, β-ketoacyl-acyl ACP synthetase I, and β-ketoacyl-acyl ACP synthetase II (D'Agnolo et al., 1975), and it is believed that a third condensing enzyme may also exist (John E. Cronan, Jr.; personal communication). β-Ketoacyl-acyl ACP synthetase I is primarily involved in unsaturated fatty acid synthesis, and the absence of this enzyme has been demonstrated in fabB unsaturated fatty acid auxotrophs (D'Agnolo et al., 1975). The type II synthetase is absent from cvc⁻ fatty acid mutants (no cis-vaccenic acid) and is proposed to be involved in elongating palmitoleoyl ACP to cis-vaccenoyl ACP (D'Agnolo et al., 1975). However, both of these enzymes display a broad preference for substrates, suggesting that either type can also participate in saturated fatty synthesis.

Since strain K1060 (a fabB mutant deficient in β-ketoacyl-acyl ACP synthetase I) underwent an ethanol-induced fatty acid change, it is unlikely that ethanol is interacting with this condensing enzyme. This leaves either the type II condensing enzyme or the putative type III condensing enzyme. In view of their broad substrate specificities, it is likely that both (or all three) condensing enzymes participate in forming the short-chained intermediates prior to the ten carbon intermediate, and the enzymes may be specific for saturated or unsaturated fatty acid synthesis only after the formation of the β-hydroxydecanoyl ACP fatty acid. Thus an inhibition of either the type II condensing enzyme or the type III

(if it exists) by ethanol would result in the reduced rate of total fatty acid synthesis observed *in vivo* as well as *in vitro*. Since the condensation reaction is the rate limiting reaction in fatty acid synthesis (Bloch and Vance, 1977), an inhibition of this reaction by ethanol would be expected to result in a corresponding decrease in the rate of overall fatty acid synthesis. Further, since the type I synthesis would already be expected to be working to its capacity, the inhibition of saturated fatty acid synthesis would probably not lead to an increase in the levels of unsaturated fatty acids. Therefore, the condensing enzyme responsible for saturated fatty acid synthesis in *E. coli* represents a logical site for the specific interaction of ethanol.

It is interesting that both ethanol and temperature were able to exert a direct effect on fatty acid synthesis *in vitro*. Our results clearly indicate that neither ethanol nor temperature depends upon the membrane, *per se*, to induce changes in *de novo* fatty acid synthesis. However, it is very possible that both effects are mediated through a lipid-protein interaction. Fatty acid synthesis is believed to occur in close proximity with the plasma membrane (van den Bosch, *et al.*, 1970), which could facilitate an association between membrane lipids and one or more of the fatty acid synthetase enzymes. Alternatively, the lipid-protein site may actually reside at the level of an enzyme-substrate interaction. This could explain our observation that ethanol induced the synthesis of shorter-chained fatty acids *in vitro* (Table 12). Both of the enzymes described above as potential sites for the ethanol interaction (β-hydroxydecanoyl ACP dehydrase and β-

ketoacyl-acyl ACP synthetase II) have also been proposed to control temperature adaptation of the fatty acid composition in E. coli (Brock et al., 1967; Gelmann and Cronan, 1972). Thus the similarity in fatty acid changes induced by ethanol and temperature may actually result from their interaction with a common enzyme in fatty acid synthesis.

REFERENCES

Bergelson, L.D., Dyatlovitskaya, E.V. and Voronkova, V.V. (1964) J. Cromatog. 15, 191-199.

Binstock, J.F., Pramanik, A., and Schultz, H. (1977) Proc. Nat. Acad. Sci. U.S.A. 74, 492-495.

Bloch, K. and Vance, D. (1977) Ann. Rev. Biochem. 46, 263-298.

Brock, D.J.H., Kass, L.R. and Bloch, K. (1967) J. Biol. Chem. 242, 4432-4440.

Chin, J.H., and Goldstein, D.B. (1977) Science 196, 684-685.

Cronan, J.E., Jr. (1974), Proc. Nat. Acad. Sci. U.S.A. 71, 3758-3762.

Cronan, J.E., Jr. (1975), J. Biol. Chem. 250, 7074-7077.

Cronan, J.E., Jr. and Wulff, D.L. (1969), Virology 38, 241-246.

Cronan, J.E., Jr. and Vagelos, P.R. (1972), Biochim. Biophys. Acta 265, 25-60.

Cronan, J.E., Jr., and Gelmann, E.P. (1975), Bact. Rev. 39, 232-256.

Cronan, J.E., Jr., Weisberg, L. and Allen, R.G. (1975), J. Biol. Chem. 250, 5835-5840.

Cubero, J.M., and Mangold, H.K. (1965), Microchem. J. 9, 227-236.

D'Agnolo, G., Rosenfeld, I.S. and Vagelos, P.R. (1975), J. Biol. Chem. 250, 5289-5294.

Esfahani, M., Barnes, E.M., and Walkil, S.J. (1969), Proc. Nat. Acad. Sci. U.S.A. 64, 1057-1064.

Fourcans, B. and Jain, M. (1974), Adv. Lipid Res. 12, 147-226.

Fox, C.F., Law, J.H., Tsukagoshi, N., and Wilson, G. (1970) Proc. Nat. Acad. Sci. U.S.A. 67, 598-605.

Freeman, C.P., and West, D. (1966), J. Lipid Res. 7, 324-327.

Gallant, J., Palmer, L. and Pao, C. (1977), Cell 11, 181-185.

Gelmann, E.P. and Cronan, J.E., Jr. (1972), J. Bacteriol. 112, 381-387.

Harder, M.E., Beacham, I.R., Cronan, J.E., Jr., Beachman, K., Honnegger, J.L., and Silbert, D.F. (1972), Proc. Nat. Acad. Sci. U.S.A. 69, 3105-3109.

Ingram, L.O. (1976), J. Bacteriol. 125, 670-678.

Ingram, L.O. (1977a), Can. J. Microbiol. 23, 779-789.

Ingram, L.O. (1977b), Appl. Environ. Microbiol. 33, 1233-1236.

Ingram, L.O., Chevalier, L.S., Gabbay, E.J., Ley, K.D., and Winters, K., (1977), J. Bacteriol. 131, 1023-1025.

Kanfer, J., and Kennedy, E.P. (1973), J. Biol. Chem. 238, 2919-2922.

Kass, L.R., Brock, D.J.H., and Bloch, K. (1967) J. Biol. Chem. 242, 4418-4431.

Klein, K., Steinberg, R., Fiethen, B., and Overath, P. (1971), Eur. J. Biochem. 19, 442-450.

Lennarz, W.J., Light, R.J. and Bloch, K. (1962) Proc. Nat. Acad. Sci. U.S.A. 48, 840-846.

Majerus, P.W., Alberts, A.W. and Vagelos, P.R. (1968) Biochemical Preparations, vol. XII, 56-65.

Marr, A.G., and Ingraham, J.L. (1962), J. Bacteriol. 84, 1260-1267.

Marsh, N.J., and Duggan, D.E. (1972), J. Bacteriol. 109, 730-740.

Merlie, J.P., and Pizer, L.I. (1973), J. Bacteriol. 116, 355-366.

Miller, J.H. (1972), Experiments in Molecular Genetics, Cold Spring Harbor, N.Y., Cold Spring Harbor Laboratory, pp. 230-234.

Morris, L.J., Wharry, D.M., and Hammond, E.W. (1967), J. Chromatog. 31, 69-76.

Nunn, W.D. (1977), Biochemistry 16, 1077-1081.

Nunn, W.D., and Cronan, J.E., Jr. (1976), J. Mol. Biol. 102, 167-172.

Okuyama, H., Yamada, K., Kameyama, Y., Ikezawa, H., Akamatsy, Y. and Nokuma, S. (1977) Biochemistry 16, 2668-2673.

Overath, P., Schairer, H.U., and Stoffel, W. (1970), Proc. Nat. Acad. Sci. U.S.A. 67, 606-612.

Rando, R.R., and Bloch, K. (1968), J. Biol. Chem. 243, 5627-5634.

Semple, K.S., and Silbert, D.F. (1975), J. Bacteriol. 121, 1036-1046.

Shaw, M., and Ingraham, J.L. (1965), J. Bacteriol. 90, 141-146.

Silbert, D.F. (1976) Methods in Membrane Biology (E.D. Korn, ed.) vol. VI, 151-182, Plenum Press, N.Y.

Silbert, D.F., Ulbright, T.M., and Honeggar, J.L. (1973a), Biochemistry 12, 164-171.

Silbert, D.F., Ladenson, R.C., and Honegger, J.L. (1973b), Biochim. Biophys. Acta. 311, 349-361.

Silbert, D.F., Pohlman, T., and Chapman, A. (1976), J. Bacteriol. 126, 1351-1354.

Sinensky, M. (1971), J. Bacteriol. 106, 449-455.

van den Bosch, H., Williamson, J.R. and Vagelos, P.R. (1970), Nature 228, 338-341.

Vogel, H.J., and Bonner, D.M. (1956), J. Biol. Chem. 218, 97-106.

Volpe, J.J., and Vagelos, P.R. (1976), Phys. Rev. 56, 339-417.

BIOGRAPHICAL SKETCH

Thomas Martin Buttke was born on December 5, 1952, in Bay Shore, New York. The author graduated from Bay Shore High School in 1970, and received the Bachelor of Arts degree from the University of North Carolina at Wilmington in 1974. In September of 1974, he entered the Department of Microbiology at the University of Florida as the recipient of a Graduate Council Fellowship, and received the Master of Science degree in microbiology in December, 1975. From September, 1977 to June, 1978, he was the recipient of a fellowship from the Center of Gerontological Studies, and he is presently a candidate for the Doctor of Philosophy degree in the Department of Microbiology and Cell Science. The author is married to the former Mary Elizabeth Thompson.

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Lonnie O. Ingram, Chairman
Associate Professor of Microbiology
and Cell Science

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

Charles M. Allen, Jr.
Associate Professor of Biochemistry

I certify that I have read this study and that in my opinion it conforms to acceptable standards of scholarly presentation and is fully adequate, in scope and quality, as a dissertation for the degree of Doctor of Philosophy.

James F. Preston III
Associate Professor of Microbiology
and Cell Science

This dissertation was submitted to the Graduate Faculty of the Department of Microbiology and Cell Science in the College of Arts and Sciences and to the Graduate Council, and was accepted as partial fulfillment of the requirements for the degree of Doctor of Philosophy.

June, 1978

Dean, Graduate School

UNIVERSITY OF FLORIDA
3 1262 08553 2702